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In Vitro Metabolism of an Insect Neuropeptide by Neural Membrane Preparations From *Lymantria dispar*[1]

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MASLER, E. P., R. M. WAGNER AND E. S. KOVALEVA. *In vitro metabolism of an insect neuropeptide by neural membrane preparations from Lymantria dispar.* PEPTIDES **17**(2) 321–326, 1996.—Neural membrane fractions, prepared from brain–subesophageal ganglion complexes of the adult lepidopteran *Lymantria dispar*, contain at least two peptidases capable of metabolizing locust adipokinetic hormone-I in vitro. The initial fragments, pGlu[1]-Leu[2]-Asn[3] and Phe[4]-Thr[5]-Pro[6]-Asn[7]-Trp[8]-Gly[9]-Thr[10], result from the action of an endopeptidase with properties similar to those reported for neutral metalloendopeptidase in *Schistocerca gregaria* and mammalian endopeptidase 24.11. The heptapeptide is further degraded by an aminopeptidase that exhibits kinetic properties similar to those described for aminopeptidase 3.4.11.2. These enzymes appear to be responsible for the first two steps in AKH catabolism in *L. dispar*.

Amastatin Aminopeptidase Bestatin Endopeptidase Enzyme assays Gypsy moth Phosphoramidon Thiorphan

THE attenuation of neuropeptide signals is regulated in large part by degradation through the action of cell surface peptidases (11). Endo- and exopeptidases work in concert to effect this degradation. A large number of these peptidases have been characterized from vertebrate tissues [see (12) for a review], and they appear to accept a broad range of substrates (8,13). Membrane-associated peptidase activities have been described in insects [*Drosophila melanogaster* (3); *Locusta migratoria* (9); *Musca domestica* (4); *Schistocerca gregaria* (1,2,10)]. Properties of the endopeptidase activity found in insects are similar to those described in the vertebrates, and the wide tissue distribution characteristic of vertebrate peptidases (8) is also seen in insects (10). The endopeptidase activity most thoroughly studied in the vertebrates and now described in insects is a metalloenzyme, endopeptidase 24.11. It is commonly associated with neural membranes (2,3), although it has been found in other tissues (10). The insect enzyme has been characterized (although not isolated) from *S. gregaria* (2,10) and its activity closely resembles that of the mammalian enzyme in substrate degradation (2) and inhibitor susceptibility (2,8,10–12). A second activity, that of an aminopeptidase, has also been reported in *S. gregaria* (2). It too appears to be membrane associated.

Information has recently become available on the nature of neuropeptide degradation in the Lepidoptera [*Lymantria dispar* (7)], and two enzyme activities have been reported. In light of the potential of neuropeptides as leads to control agents (5), and the importance of lepidopterans to agricultural economy, we examined neuropeptide degradation in *L. dispar*, and characterized endo- and aminopeptidase activities.

METHOD

Insect Rearing and Culture

Origins of the culture of *L. dispar* used in this study have been described previously (6). A diapausing strain was used and eggs were chilled (5–7°C, 180 days) to break diapause. Eggs were transferred to a rearing chamber and held at 25°C, 50–60% relative humidity, and 16 h:8 h L:D photoperiod. Larvae were hatched and maintained on a high wheat germ diet. Under these conditions, pupation occurs in approximately 4 weeks. Pupae were segregated by size (females are distinctly larger than males), and adults, which eclose 10–14 days after pupation, were collected daily and held at the conditions described above.

Tissue Collection

Animals were anesthetized with carbon dioxide. Heads were removed, cleaned of hairs by gently rubbing on a moist paper

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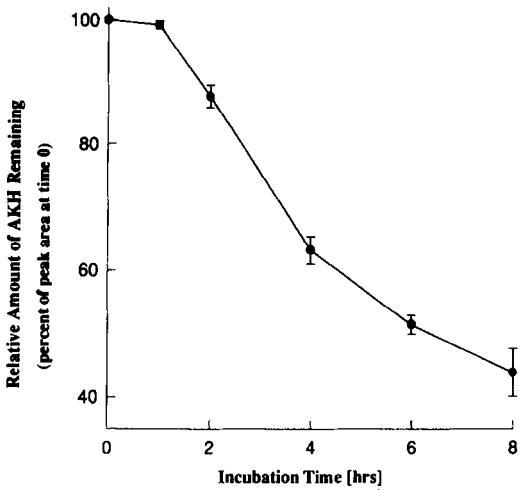

FIG. 1. Time course of AKH digestion by neural membrane preparations from *L. dispar*. Neural membrane preparations (P_3) were incubated with locust adipokinetic hormone (AKH) for the indicated times. Incubations were fractionated by RP-HPLC and eluting peaks were monitored at 214 nm. Peak areas were calculated by peak integration and the relative areas of the AKH peak are plotted vs. incubation time. Peak areas are expressed as a percent of the peak area of AKH in nonincubated (i.e., 0 time) mixtures. Each data point represents the mean ± SE of four separate determinations.

towel, and the brain–subesophageal ganglion complex (brain-SEG) removed through a dorsal cut made in the head. Complexes were either collected in 2-ml polypropylene tubes on ice for immediate homogenization, or were immediately frozen by collecting in tubes on dry ice and stored at −80°C.

Enzyme Preparation

Tissue was homogenized in 50 m*M* Tris-HCl, pH 7.3, at one tissue equivalent per 40 μl, on ice, using a Polytron (Brinkman Instruments, NY) fitted with a PT-7 generator. The homogenate was centrifuged for 10 min at 4°C and the 1000 × *g* supernatant (S_1) collected, then centrifuged at 30,000 × *g* for 20 min at 4°C. The resulting pellet (P_2) was suspended (1 tissue equivalent/40 μl) in 50 m*M* Tris-HCl, pH 7.3, containing 0.5 *M* NaCl. The suspension was centrifuged at 30,000 × *g* for 20 min at 4°C, and the pellet (P_3) was suspended in 100 m*M* Tris-HCl, pH 7.3 (1 tissue equivalent/40 μl) for enzyme assay. The effect of pH on enzyme activity was evaluated in duplicate experiments by preparing P_3 in buffer at each of four pH levels (5.3, 6.3, 7.3, 8.3). The percent degradation of AKH after 6 h of incubation was 33, 26, 52, and 13 for pH 5.3, 6.3, 7.3, and 8.3, respectively. Consequently, all extractions and assays were done using the pH 7.3 buffer.

Enzyme Assays

For the chromatographic assay, digestions were done in a total of 50 μl in 100 m*M* Tris-HCl, pH 7.3, at 37°C. The insect peptide neurohormone locust adipokinetic hormone-I (AKH-I; Peninsula Laboratories, Belmont, CA) was used as substrate at 4.3×10^{-5} *M* final concentration, and tissue extract concentration was typically ca. 10 μg/brain-SEG equivalent/assay. Incubations were stopped by the addition of 100 μl of 60% acetonitrile (CH_3CN, Fisher Scientific, Springfield, VA) in 0.1% trifluoroacetic acid (TFA, Aldrich Chemical Co., Milwaukee, WI), then stored at −80°C. Specific inhibitors used were amastatin {[(2*S*,3*R*)-3-amino-2-hydroxy-5-methyl-hexanoyl]-Val-Val-Asp-OH; aminopeptidase inhibitor} at 1×10^{-3} *M* final concentration, phosphoramidon [*N*-(α-rhamnopyranosyloxy-hydroxyphosphinyl)-Leu-Trp-OH; metalloendopeptidase inhibitor] at 1×10^{-3} *M*, or less, final concentration, and thiorphan (DL-3-mercapto-2-benzyl-propanoylglycine; endopeptidase inhibitor) at 1×10^{-3} *M*, or less, final concentration. Each was from Sigma Chemical Co. (St. Louis, MO). Inhibitors were incubated with extracts (enzyme) for 10 min at 37°C prior to addition of the substrate. Substrate and cleavage products were resolved through fractionation by reverse-phase HPLC (RP-HPLC). The system comprised a Delta Pak C18 column (3.9 × 150 mm, Millipore Corp., Milford, MA) with a linear gradient of 3.33% CH_3CN/min in 0.1% TFA over 15 min at a flow rate of 0.78 ml/min. Absorbance was monitored at 214 nm, peaks were identified by retention time, and relative abundance was estimated by peak area integration. Selected peaks were analyzed by automated Edman degradation using an Applied Biosystems Model 477A pulsed liquid-phase sequencer with HPLC analysis on-line with a Model 120A phenylthiohydantoin (PTH) analyzer (Applied Biosystems, Inc., San Jose, CA).

For the colorimetric assay, incubations of 250 μl final volume were done in 96-well polystyrene microtiter plates (Corning Easy Wash Assay plates, Corning, NY). The P_3 samples were prepared as above and dissolved in 2 m*M* Tris, pH 7.3, at 1 brain-SEG equivalent/40 μl buffer. For incubations, 40 μl of P_3 and 206 μl of buffer were combined in a microtiter well. The reaction was started with the addition of 4 μl of substrate [L-alanine-4-nitroanilide-HCl (Fluka Chemical Co., Ronkonkoma, NY) dissolved in HPLC-grade methanol at a concentration of 667 nmol/μl]. Changes in absorbance were monitored at 414 nm with a Titertek MC10 microplate reader (Flow Laboratories, McLean, VA). Under these conditions, the reaction was linear for at least 60 min. The reaction product, *p*-nitroaniline, absorbs 7.44×10^{-3} absorbance units at a concentration of 1 nmol/ml. This value was used to convert changes in absorbance in reaction mixtures into nmol of product generated. The inhibitors amastatin and bestatin {[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-bu-

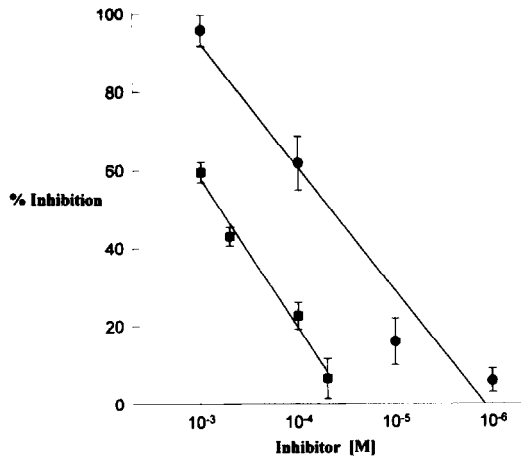

FIG. 2. Effect of metalloendopeptidase inhibitors on the degradation of AKH by *L. dispar* neural membrane preparations. Membrane preparations (P_3) and AKH were incubated for 6 h with various concentrations of phosphoramidon (■) or thiorphan (●). Reaction mixtures were fractionated by RP-HPLC and peak areas estimated by integration as described. AKH peak areas from incubations containing inhibitor were compared with peak areas from parallel incubations with no inhibitor. Differences in peak areas are expressed as percent inhibition. Each data point represents the mean ± SE of three to eight separate determinations.

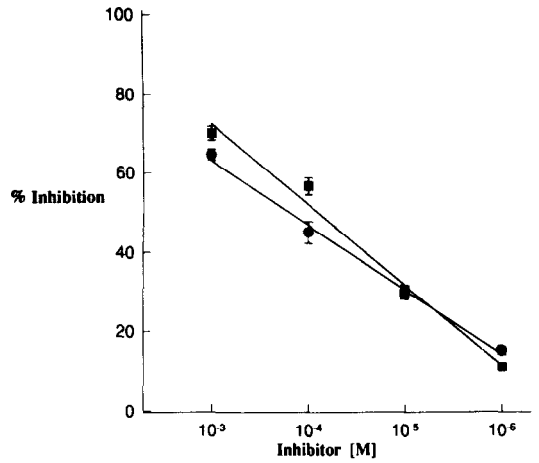

FIG. 3. Effect of inhibitors on aminopeptidase activity in *L. dispar* neural membrane preparations. Membrane preparations (P_3) were incubated with the colorimetric substrate L-alanine-4-nitroanilide-HCl, and various concentrations of the inhibitors amastatin (●) or bestatin (■), as described in the Method section. Final concentration of inhibitors ranged from 1×10^{-3} *M* through 1×10^{-6} *M*. Reactions were monitored by increase in absorbance at 414 nm. Each data point represents the mean ± SE of three separate determinations at the inhibitor dose indicated.

tanoyl]-Leu-OH; aminopeptidase inhibitor; from Sigma} were each dissolved and diluted in HPLC grade H_2O. Inhibitor (5 μl) was combined with 40 μl of P_3, 201 μl of buffer, and incubated for 15 min. Substrate (4 μl) was then added and the reaction monitored at 414 nm. Final concentrations of inhibitors used were 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, and 1×10^{-6} *M*. The reaction was linear for at least 60 min. Percent inhibition was calculated by comparing the change in absorbance over 60 min of the reaction in the presence and absence (control) of inhibitor.

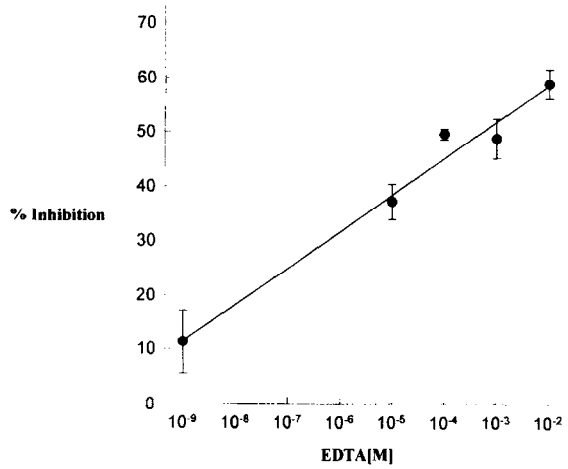

FIG. 4. Effects of a chelating agent on the degradation of AKH by *L. dispar* neural membrane preparations. Membrane preparations (P_3) and AKH were incubated for 6 h with various concentrations of EDTA. Reaction mixtures were fractionated by RP-HPLC and peak areas estimated by integration as described. AKH peak areas from incubations containing EDTA were compared with peak areas from parallel incubations with no EDTA. Differences in peak areas are expressed as percent inhibition. Each data point represents the mean ± SE of three separate determinations.

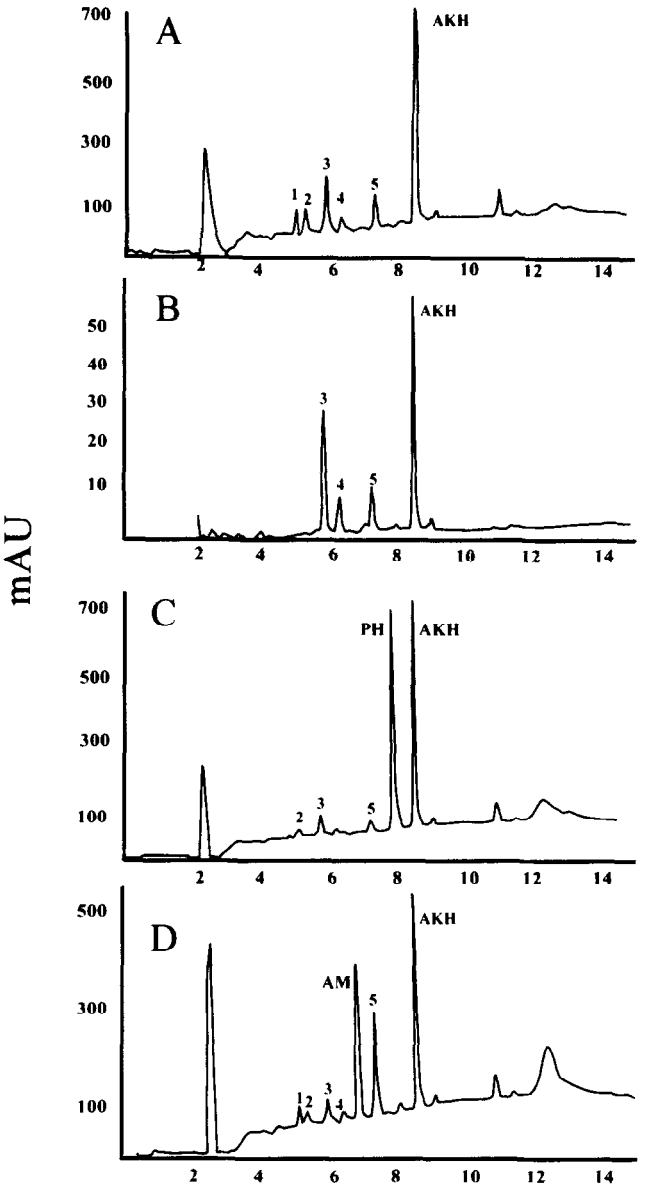

FIG. 5. Chromatographic profiles of AKH digests. Neural membrane preparations (P_3) were incubated for 6 h with locust adipokinetic hormone (AKH) (A,B) or with AKH plus phosphoramidon (C) or AKH plus amastatin (D). Following incubation, the mixtures were fractionated by RP-HPLC and monitored at either 214 or 280 nm. Peaks 1 and 3–5 were identified by amino acid sequencing (see Table 1). AM = amastatin; PH = phosphoramidon. (A) membrane preparation (P_3) + AKH, 214 nm trace; (B) P_3 + AKH, 280 nm trace; (C) P_3 + AKH + 10^{-3} *M* phosphoramidon, 214 nm trace; (D) P_3 + AKH + 10^{-3} *M* amastatin, 214 nm trace.

Protein Determination

Total protein levels in extracts were estimated using the Bio-Rad Protein Assay (Bio-Rad, Inc., Richmond, CA) modified for use in 96-well microtiter plates (200 μl total volume/well). Absorbance at 595 nm was measured with the Titertek plate reader.

RESULTS

Using integrated peak areas as a measure of peptide level, just under 40% of AKH was lost after 4 h of incubation and nearly

TABLE 1

PEPTIDE FRAGMENTS RESULTING FROM THE DIGESTION OF AKH BY *L. dispar* NEURAL MEMBRANE PREPARATIONS

Peak No.*	RT (min)†	Sequence
1	4.8	pGlu^1-Leu^2-Asn^3
2	5.2	?‡
3	5.7	Asn^7-Trp^8-Gly^9-Thr^{10}
4	6.2	Thr^5-Pro^6-Asn^7-Trp^8-Gly^9-Thr^{10}
5	7.2	Phe^4-Thr^5-Pro^6-Asn^7-Trp^8-Gly^9-Thr^{10}
AKH	8.4	pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2

Incubations were conducted for 6 h, then fractionated by RP-HPLC. Peaks were collected and then identified by amino acid sequencing. See the Method section for details.

* Peaks are numbered by order of elution.

† Retention time (RT) was recorded at the maximum peak absorbance.

‡ The sequence of this fragment was not unambiguously determined.

TABLE 2

INFLUENCE OF INHIBITORS ON THE RELATIVE LEVELS OF SUBSTRATE (AKH) AND PRODUCTS (METABOLITE PEAKS) FOLLOWING *IN VITRO* INCUBATION

	Percent Peak Area (±SE)*		
Peak†	Control‡	Amastatin‡	Phosphoramidon‡
AKH	48.9 ± 1.3	48.5 ± 1.4	80.7 ± 0.8
1	4.3 ± 1.4	3.7 ± 1.1	0.4 ± 0.4
2	4.9 ± 1.4	2.7 ± 0.6	0.7 ± 0.6
3	15.2 ± 4.0	5.8 ± 0.7	7.9 ± 0.8
4	8.9 ± 1.7	4.6 ± 0.4	3.0 ± 0.7
5	16.5 ± 4.5	32.4 ± 1.3	6.8 ± 1.4

* Following incubation, samples were chromatographed on RP-HPLC (both procedures described in the Method section) and elution profiles monitored at 214 nm. Peak areas were determined by integration and each value computed as a percent of total integrated peak area. Each value represents the mean ± SE for four to six replicates.

† Peaks are named by order of elution (except AKH), and are identified in Table 1. Typical chromatograms are shown in Fig. 5.

‡ Prior to addition of substrate and incubation (see the Method section for details), the enzyme preparation (P_3) was incubated for 10 min, 37°C, in the presence of amastatin or phosphoramidon (1 m*M* each), or with no inhibitor (control).

50% was lost after 6 h (Fig. 1). Although the 4-h digest was adequate to observe AKH loss, we chose 6-h as a standard incubation time to optimize the integration of the metabolite peaks, which were much smaller than the AKH peak (Fig. 5).

Phosporamidon and thiorphan each showed dosage-dependent inhibition of AKH degradation (Fig. 2), although thiorphan was over 11-fold more potent. Slight inhibition (approximately 10%) occurred at 10^{-6} *M* thiorphan (circles) and maximum inhibition (95%) was achieved at 10^{-3} *M*. The IC_{50} is 6×10^{-5} *M*. With phosphoramidon (squares), some inhibition (approximately 10%) was noted at 5×10^{-5} *M*, and 60% at 10^{-3} *M*, the maximum concentration tested. The IC_{50} is 7×10^{-4} *M*. Molar dose ranges, from minimum to maximum observed inhibition, are 10^3 for thiorphan and $10^{1.5}$ for phosphoramidon.

Aminopeptidase activity was inhibited by either amastatin or bestatin (Fig. 3), although bestatin showed approximately 2.5-fold more potency. Using the colorimetric assay, inhibition at the maximum dose tested (10^{-3} *M*) was 65% for amastatin and 70% for bestatin. IC_{50} concentrations were 2×10^{-4} and 8×10^{-5} *M* for amastatin and bestatin, respectively, and each showed a 10^3 molar dose range.

The metal chelating agent EDTA inhibited the degradation of AKH by nearly 60% at 10^{-2} *M* with an IC_{50} at 5×10^{-4} *M* (Fig. 4). At 10^{-9} *M*, inhibition was just over 10%. The molar dose range, from minimum to maximum observed inhibition, is 10^7.

Five metabolite peaks, all absorbing at 214 nm, eluted between 4 and 8 min [Fig. 5(A)]. The sequences for four of the five are shown in Table 1. Peak 1 (pGlu^1-Leu^2-Asn^3) does not absorb at 280 nm [Fig. 5(B)], whereas peaks 3 (Asn^7-Trp^8-Gly^9-Thr^{10}), 4 (Thr^5-Pro^6-Asn^7-Trp^8-Gly^9-Thr^{10}), and 5 (Phe^4-Thr^5-Pro^6-Asn^7-Trp^8-Gly^9-Thr^{10}) absorb strongly [Fig. 5(B)]. Peak 2, which was not unambiguously identified (Table 1), does not absorb at 280 nm [Fig. 5(B)]. A maximum dose of phosphoramidon (10^{-3} *M*) greatly reduced the levels of all five metabolite peaks [Table 2, Fig. 5(C)], but did not completely eliminate them. The loss of AKH was reduced in the presence of phosphoramidon. AKH accounted for 80% of total peak area following incubation with 10^{-3} *M* phosphoramidon vs. less than 50% in either control incubations or in the presence of 10^{-3} *M* amastatin (Table 2). Amastatin, even at the maximum dose tested (10^{-3} *M*), did not affect the degradation of AKH, but did enhance the accumulation of peak 5 [Table 2, Fig. 5, compare (A) with (D)]. The levels of peaks 1–4 were reduced, to varying degrees, in the presence of amastatin [Table 2, Fig. 5, compare (A) with (D)]. The accumulation of peak 5 (Phe^4-Trp^5-Pro^6-Asn^7-Trp^8-Gly^9-Thr^{10}) is further illustrated in Fig. 6. In the absence of amastatin, peak 5 accounted for a maximum of 10% total peak area after 4 h of incubation. In the presence of 10^{-3} *M* amastatin, however, peak 5 accumulated to over 30% total peak area at 4 h, and continued to accumulate through 6 h. Note that amastatin had no effect on the rate of disappearance of AKH.

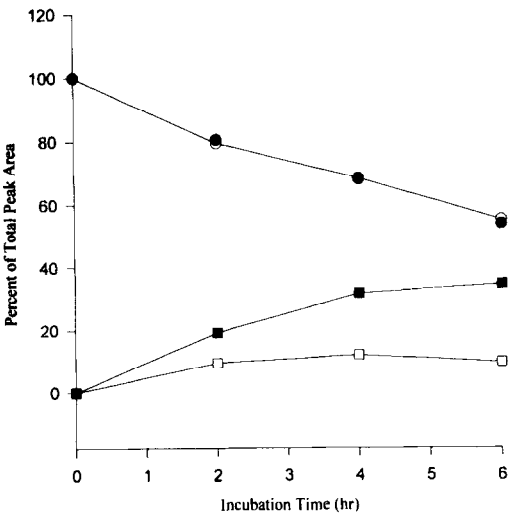

FIG. 6. Effect of amastatin on the degradation of AKH. Neural membrane preparations (P_3) were incubated with AKH and 10^{-3} *M* amastatin. At the times indicated, the incubations were stopped by the addition of 60% CH_3CN/0.1% TFA and processed as described in the Method section. Peak areas were determined by integration and data are expressed as the percent of the integrated area of the AKH peak (●) or peak No. 5 (■), in the presence (● ■) or absence (○ □) of amastatin, relative to the total peak area.

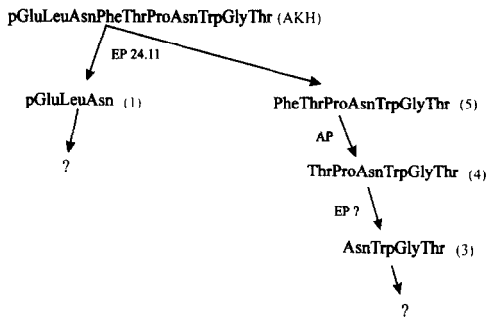

FIG. 7. Model for the degradation of AKH by *L. dispar* neural membrane preparations. Neural membrane preparations (P_3) were incubated for 6 h in the presence of AKH and the presence or absence of inhibitor (phosphoramidon; amastatin). Peptide fragments were identified by fractionation on RP-HPLC followed by amino acid sequencing. EP = endopeptidase; EP24.11 = endopeptidase 24:11; AP = aminopeptidase; ? = peptide or enzyme unknown.

DISCUSSION

Endopeptidase and aminopeptidase activities were detected in neural membrane preparations from brain–subesophageal ganglion complexes obtained from adult female *L. dispar*. Incubation of locust adipokinetic hormone with the membrane preparation resulted in the appearance, at 214 nm, of 5 UV peaks following RP-HPLC [Fig. 5(A)]. Analysis of each peak by amino acid sequencing lead to the unambiguous identification of peaks 1, 3, 4, and 5 (Table 1). Equivocal results from the attempted sequencing of peak 2 prevented its identification. However, its absence from the UV profile at 280 nm [Fig. 5(B)] suggests that it may be an *N*-terminal fragment. The endopeptidase activity was similar to that reported for endopeptidase 24.11, a neutral metalloendopeptidase (12), which cleaves at the amino side of hydrophobic residues such as phenylalanine, and is specifically inhibited by phosphoramidon or thiorphan (8). Each of these inhibitors decreased the rate of AKH degradation in the *L. dispar* system (Fig. 2). The response of *L. dispar* AKH-degrading activity to concentrations of phosphoramidon in the 10^{-3} to 10^{-5} *M* range is consistent with results obtained in other insect systems. Phosporamidon, at 7×10^{-4} *M*, inhibits endopeptidase activity 50% in *L. dispar* neural membrane preparations. This compares with 1×10^{-4} *M*, which inhibited activity 50% in *S. gregaria* crude membrane preparations (2), and 2×10^{-5} *M*, which inhibited AKH metabolism by 65% in *S. gregaria* flight muscle membrane preparations (10). Significantly better inhibition was obtained with *M. domestica* head membrane preparations (4), which yielded IC_{50} values of 2.5×10^{-7} and 2.5×10^{-6} *M*, respectively, for phosphoramidon and thiorphan. This may have been a result of more extensive washing of the membrane preparations, resulting in a "cleaner" preparation than ours. In an in vitro assay for allatostatin inhibition of juvenile hormone production by the corpora allata, the addition of phosphoramidon at greater than 10^{-5} *M* significantly increased the efficacy of the allatostatin (14). Presumably, endopeptidase activity (i.e., endopeptidase 24.11) was inhibited, increasing the half-life of allatostatin in vitro. Our inability to completely inhibit AKH degradation (at 10^{-3} *M* phosphoramidon, inhibition was 60%, Fig.2) suggests that, not surprisingly, other endopeptidases that can use AKH as a substrate are present in these crude membrane preparations. In fact, crude neural membrane preparations from *S. gregaria* contain both synaptic and mitochondrial membranes and the majority of AKH-degrading activity is associated with the synaptic fraction (2). This synaptic membrane endopeptidase was inhibited by phosporamidon, whereas the mitochondrial fraction was relatively insensitive to phosporamidon. Thiorphan, also an inhibitor of endopeptidase 24.11 (2,8), inhibited AKH-degrading activity in our system, and was more effective than phosphoramidon (IC_{50} was 6×10^{-5} *M*, Fig. 2). EDTA inhibited 50% at 10^{-3} *M* and 40% at 10^{-5} *M* (Fig. 4), indicating that a significant portion of the AKH-degrading activity in *L. dispar* neural membrane preparations is due to a metalloenzyme.

Based upon the cleavage site preferences of endopeptidase 24.11 (8), and the fate of AKH when exposed to *S. gregaria* membrane preparations (2,10), one would predict that the Asn^3-Phe^4 bond would be a key cleavage site. This appears to be the case in our system, as $pGlu^1$-Leu^2-Asn^3 (peak 1) and Phe^4-Thr^5-Pro^6-Asn^7-Trp^8-Gly^9-Thr^{10} (peak 5) are major products of AKH digestion in *L. dispar* [Fig. 5(A), Table 1]. Further, the presence of amastatin caused the distinct accumulation of peak 5 [Fig. 5(D), Fig. 6]. This not only indicates that an aminopeptidase is present in *L. dispar* neural membrane preparations, but supports the conclusion that the initial cleavage of AKH is at the Asn^3-Phe^4 bond. Based upon these observations, and the identity of the peptide fragments produced, we propose a tentative pathway for the metabolic fate of AKH in *L. dispar* (Fig. 7). Cleavage of the Asn^3-Phe^4 bond yielded peaks 1 and 5. Further degradation of peak 1 was not assessed. Peak 5 was further processed by an aminopeptidase to yield peak 4, which lacks the *N*-terminal phenylanine of peak 5. The generation of peak 3 (Asn^7-Trp^8-Gly^9-Thr^{10}) may have been the result of sequential action by the aminopeptidase, or the action of other endopeptidases as yet not identified. This model is similar to one proposed by Rayne and O'Shea (10) for the explanation of their observations regarding AKH metabolism in circulating hemolymph of *S. gregaria*. In their system, Trp^8-Gly^9-Thr^{10} was identified as a metabolite of Asn^7-Trp^8-Gly^9-Thr^{10}. We did not detect this tripeptide fragment. It is probably not identical to our peak 2 because it contains tryptophan, which would absorb at 280 nm. It is interesting that the fragment Pro^6-Asn^7-Trp^8-Gly^9-Thr^{10} was not identified in either system [(Fig. 7; (10)], even though two different methods of detection were used (amino acid sequencing vs. radiolabeling).

REFERENCES

1. Isaac, R. E. Proctolin degradation by membrane peptidases from nervous tissues of the desert locust (*Schistocera gregaria*). Biochem. J. 245:365–370; 1987.
2. Isaac, R. E. Neuropeptide degrading endopeptidase activity of locust (*Schistocerca gregaria*) synaptic membranes. Biochem. J. 255:843–847; 1988.
3. Isaac, R. E.; Priestly, R. M. Neuropeptide metabolism: Properties of an endopeptidase from heads of *Drosophila melanogaster*. In: Borkovec, A. B.; Masler, E. P., eds. Insect neurochemistry and neurophysiology 1989. Clifton, NJ: Humana Press; 1990:267–270.
4. Lamango, N. S.; Isaac, R. E. Metabolism of insect neuropeptides: Properties of a membrane bound endopeptidase from heads of *Musca domestica*. Insect Biochem. Mol. Biol. 23:801–808; 1993.
5. Masler, E. P.; Kelly, T. J.; Menn, J. J. Insect neuropeptides: Discovery and application in insect management. Arch. Insect Biochem. Physiol. 22:87–111; 1993.
6. Masler, E. P.; Bell, R. A.; Thyagaraja, B. S.; Kelly, T. J.; Borkovec, A. B. Prothoracicotropic hormone-like activity in the embryonated eggs of gypsy moth, *Lymantria dispar* (L.). J. Comp. Physiol. [B] 161:37–41; 1991.

7. Masler, E. P.; Wagner, R. M. Metabolic enzymes of bioactive neuropeptides in gypsy moth, *Lymantria dispar*. In: Borkovec, A. B.; Loeb, M. J., eds. Insect neurochemistry and neurophysiology 1993. Boca Raton, FL: CRC Press; 1994:329–332.
8. Matsas, R.; Kenny, A. J.; Turner, A. J. The metabolism of neuropeptides. Biochem. J. 223:433–440; 1984.
9. Puiroux, J.; Loughton, B. G. Degradation of the neuropeptide proctolin by membrane bound proteases of the hindgut and ovary of *Locusta migratoria* and the effects of different inhibitors. Arch. Insect Biochem. Physiol. 19:193–202; 1992.
10. Rayne, R. C.; O'Shea, M. Inactivation of neuropeptide hormones (AKHI and AKHII) studied in vivo and in vitro. Insect Biochem. Mol. Biol. 22:25–34; 1992.
11. Turner, A. J. Neuropeptide signaling and cell-surface peptidases. In: Nishizuka, Y., et al., eds. The biology and medicine of signal transduction. New York: Raven; 1990:467–471.
12. Turner, A. J.; Hooper, N. M.; Kenny, A. J. Neuropeptide-degrading enzymes. In: Fink, G.; Harmar, A. J., eds. Neuropeptides: A methodology. London: John Wiley and Sons; 1989:189–223.
13. Turner, A. J.; Matsas, R.; Kenny, A. J. Are there neuropeptide-specific peptidases? Biochem. Pharmacol. 34:1347–1356; 1985.
14. Yagi, K. J.; Yu, C. G.; Tobe, S. S. Phosphoramidon enhances allatostatin-mediated inhibition of juvenile hormone biosynthesis in the corpora allata of the cockroach, *Diploptera punctata*. Experientia 48:758–761; 1992.